Responses of grapevine cells to physiological doses of ethanol, among which induced resistance to heat stress

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Abbreviations :

DEGs – Differentially Expressed Genes

EtOH – Ethanol

GO – Gene Ontology

HS – Heat Shock

HSP – Heat-Shock Protein

PCA – Principal Component Analysis

RNA-seq - RNA sequencing

sHSP – small Heat-Shock Protein

Abstract

Grapevine naturally endures stresses like heat, drought, and hypoxia. A recent study showed very low oxygen levels inside grape berries, linked to ethanol content. Other studies have established the link between ethanol and tolerance to various stresses: heat stress, drought, and high salinity. The causes

of such a tolerance are not well understood. In our study, three-week-old Gamay calli, *Vitis vinifera*, were characterised for their endogenous oxygen levels and endogenous ethanol concentration. Subsequently, a transcriptomic study of these cells was conducted, 6 and 24 hours after treatment with 1 mM ethanol. After 6 hours, ethanol addition led to 386 differentially expressed genes, with a notable upregulation of genes related to heat response, especially small Heat Shock Proteins (sHSPs). Further experiments showed that ethanol priming in grape cells or in *Arabidopsis* seedlings reduced pigment and electrolyte leakage under heat stress, respectively. This study supports the idea that ethanol priming helps protect plants against heat stress and provides a valuable RNA-seq dataset for further research into the underlying mechanisms, sHSPs playing a potentially crucial role in this adaptive response.

Introduction

Hypoxia occurs in plant cells due to the insufficient availability of oxygen (Loreti and Perata, 2020). Two types of hypoxia can be distinguished: environmental hypoxia can be caused by specific environmental cues, while developmental hypoxia occurs in tissues and organs in well-oxygenated surroundings, under normal atmosphere oxygen availability (Jethva *et al*., 2022).

Environmental hypoxia is a condition periodically encountered by terrestrial plants, often in waterlogged soils (Daniel and Hartman, 2024). This phenomenon can also be induced by rising temperatures, as dissolved oxygen levels decrease with higher temperatures (Chapra *et al*., 2021) and increased microbial activity further depletes oxygen (Alkorta *et al*., 2017). Additionally, hypoxia occurs at high altitudes due to the reduction in oxygen partial pressure (Abbas *et al*., 2022).

Developmental hypoxia naturally occurs in bulky or dense tissues like seeds, fruits, teguments, and roots where oxygen diffusion is limited (Brazel and Graciet, 2023). It also occurs during rapid cell proliferation when the respiratory rate is intrinsically high: oxygen is consumed faster than it diffuses into the plant tissue (Geigenberger, 2003). For instance, Xiao *et al* (2018) reported very low levels of oxygen in grape berries during growth and late-ripening processes. Furthermore, developmental hypoxia has been observed in growing potato tubers (Geigenberger *et al*., 2000), in the embryos of faba bean (*Vicia faba*) and green pea (*Pisum sativum*) (Rolletschek *et al*., 2002), and in seeds during the germination process (Borisjuk and Rolletschek, 2009). Oxygen levels have been observed to decrease to hypoxic levels during rapid cell proliferation in specific organs, such as the shoot apical meristem and developing monocot anthers in *Zea mays* (Dukowic-Schulze and van der Linde, 2021), and during grapevine bud burst (Meitha *et al*., 2018).

Low oxygen concentrations induce cellular stress due to decreased ATP production, depletion of energy reserves, and accumulation of metabolic end-products notably ethanol (Jethva *et al*., 2022)

69 through fermentative metabolism (Raymond *et al*., 1995). Ethanol also accumulates during particular
70 plant developmental stages listed above: embryo and seedling development, fruit development, and
71 maturation (Diot *et al*., 2024). It also accumulates in response to many other stresses including air
72 pollution (sulphur dioxide and ozone exposure), water deficit, and freezing (Kimmerer and Kozlowski,
73 1982).

74 The impact of ethanol on plant physiology, particularly at low doses, is an emerging area of
75 research as it could be a potential molecular marker of hypoxia (Diot *et al*., 2024). While high
76 concentrations of ethanol are known to be toxic to plants, the few studies having investigated the
77 effects of low physiological doses of ethanol on plants show beneficial outcomes, like resistance to
78 heat stress (Matsui *et al*., 2022; Todaka *et al*., 2024), resistance to drought (Bashir *et al*., 2022) or
79 resistance to high salinity (Das *et al*., 2022).

80 Matsui *et al*. (2022) have shown that exposure to physiological doses of ethanol modulates
81 Heat Shock Protein (HSP) expressions in *Arabidopsis* seedlings. HSPs are molecules known to be
82 expressed in response to a wide range of stresses, including thermal, chemical, and oxidative stress
83 (Park and Seo, 2015). Among these HSPs, there is a subfamily called small heat shock proteins (sHSPs)
84 which is proposed to act as molecular chaperones to protect other proteins from stress-induced
85 damage (Waters and Vierling, 2020). The relationship between hypoxia and HSPs has already been
86 studied, and it has been shown that the activation of HSPs is critical to adaptation to hypoxia and for
87 enduring the oxidative stress of reoxygenation (Baird *et al.,* 2006). Chen *et al*., (2014) further showed
88 that HSP70 accumulated more than 10 folds in the soybean plasma membrane in response to hypoxia
89 stress. However, none of these studies established the link between ethanol, hypoxia and HSP.
90 Moreover, most studies on ethanol investigate organisms' response to "hammer" doses of this simple
91 alcohol, i.e. non-physiological doses.

92 In the present study, we investigate the impact of ethanol at a physiological dose, 1 mM, on
93 gene transcript expressions in grapevine cell cultures, as Xiao *et al*. (2018) have shown that hypoxic
94 conditions occurred during grape berry development. Since the late 1970s, it has been widely accepted
95 that plant cell cultures have numerous applications, among which serving as an excellent system for
96 studying plant cell genetics, physiology, biochemistry, and pathology (Smetanska, 2008). However,
97 there is a noticeable gap in the literature regarding the characterization of cell cultures grown on solid
98 media. Indeed, most research focuses on plant cell suspension cultures (Hellwig *et al*., 2004; Sato,
99 2013), rather than on plant cell cultures grown on agar-solidified medium in Petri dishes (i.e., calli).

100 After characterising the physiological endogenous concentrations of oxygen and ethanol in
101 these cell cultures, we carried out a whole-genome transcriptomic study using RNA-seq. Among the
102 gene families up-regulated by a physiological dose of ethanol treatment, we identified genes encoding
103 for HSPs. Following this observation, a series of metabolite leakage measurements confirmed the

104 protective role of ethanol priming on grapevine cells exposed to heat shock, initially shown by Matsui
105 *et al*. (2022) in lettuce leaves.
106
107
108 **Materials and methods**

109 Plant materials

110 Experiments were performed on a grapevine model. Cells from the berry of *Vitis vinifera* L. cv.
111 Gamay Fréaux were sub-cultured in 55x15 mm Petri dishes using a previously published method
112 (Triantaphylidès *et al*., 1993), with modifications concerning the culture medium. The composition of
113 the growth medium used in this study is given in Supplementary Table S1. Parafilm was wrapped
114 around the plates to prevent the medium and the calli from drying out. The plates were then placed
115 in a climatic chamber (CRYO RIVOIRE, France) under constant conditions with a day/night photoperiod
116 of 16h/ 8h, a temperature of 25°C/ 20°C and a light intensity of 150 µmol.m-1.s-1. Plates were moved
117 every day within the chamber to ensure homogenous growth conditions.
118 A complementary experiment was performed on *Arabidopsis thaliana* seedlings var. Col-0.
119 Seeds were sterilised using a 4.8% bleach solution for 10 minutes. Following sterilisation, the seeds
120 were sown individually onto 35x15 mm Petri dishes containing MS/2 medium with 0, 0.1 or 1 mM
121 ethanol (EtOH), using a sterile toothpick for placement. The Petri dishes were then sealed with
122 micropore surgical tape (3M, 1.25 cm width, Germany) and stratified at 4°C for 24 hours. After
123 stratification, the plates were transferred to a growth chamber (CRYO RIVOIRE, France) where the
124 seeds were cultivated for 11 days under controlled conditions: light/dark, 25/20°C, 14h/10h and 150
125 µmol.m-1.s-1. Plates were moved every day within the chamber to ensure homogenous growth
126 conditions.
127

128 Characterization of cell cultures of *Vitis vinifera* L. cv. Gamay Fréaux.

129 Callus development monitoring: Calli were grown for five weeks and photographed at 0, 7, 14,
130 21, 28 and 35 days in Petri dishes. The selected calli shown in the results were representative of the
131 observed general development. For each time point, the average callus weight has been measured
132 using a microscale. Calli grown for 14 and 35 days were harvested for cross-sections and photographed
133 using the Axio Zoom.V16 microscope (ZEISS, Germany).
134 Endogenous ethanol measurements: For different stages, calli were frozen in liquid nitrogen.
135 Each callus was ground to a fine powder and aliquoted at 200 mg/sample. The samples were then
136 thawed in an Eppendorf 5415R centrifuge (Eppendorf, Germany) at 4°C and 13,600 g for 10 minutes.
137 The supernatant was recovered and assayed for ethanol content using an enzymatic assay by
138 measuring NADH absorbance at 340 nm (Ethanol kit, BioSentec, France)

139 Endogenous oxygen measurements: Dissolved oxygen in grapevine calli was measured using a
140 Clark O_2 microelectrode with a 25 µm diameter tip (OX-25; Unisense A/S, Denmark). The
141 microelectrode was calibrated in 0% oxygen solution (0.1 M NaOH, 0.1 M $C_6H_7NaO_6$) and in fully
142 oxygenated water (273 µmol.L^{-1} at 22°C) as 100% O_2 solution. Individual callus (equilibrated to room
143 temperature) was placed on a motorised micromanipulator. The microelectrode was placed in the
144 middle of the callus and [O_2] profiles were taken in depth towards the centre of the callus at 300 µm
145 increments. Oxygen readings were recorded using Unisense Suite software (Unisense A/S).
146 Measurements were performed using 4 calli for each week of development.
147

148 ## EtOH treatment and sampling for RNA sequencing

149 We selected the callus stage (i.e. 21 days, Fig. 1A) to have sufficient weight of plant material
150 for the RNA extractions and because calli were in a healthy growing phase. Considering the
151 endogenous ethanol levels in the callus at 21 days (406 ± 2 µM, Fig. 1C), we chose to treat our calli
152 with 1 mM EtOH for the experiment. This treatment represents a 13% increase in ethanol
153 concentration based on average callus weight and the volume of the treatment solution (see
154 Supplementary Table S2). Due to the limited research on this subject, two sampling times were chosen:
155 6 hours and 24 hours post-treatment, to cover what we called short and long-term responses. Cell
156 cultures were thus treated with either 100 µL of a control solution (fresh growth medium) or with 100
157 µL of a fresh growth medium containing 1 mM EtOH; and then placed back in their culture chamber
158 for either 6 hours or 24 hours. Growth culture medium for this treatment purpose was prepared
159 without agar. After the incubation time, each callus was then collected in 10 mL Falcon™ tubes and
160 immediately immersed in liquid nitrogen, then stored at -80°C.
161

162 ## RNA extraction and purification

163 Frozen callus samples were homogenised to powder with the ball grinder Tissue Lyser II
164 (Qiagen, Germany) by applying one cycle of 2 minutes at 300 Hz. Total RNA from individual calli was
165 extracted using 160 mg of frozen powder with the Promega ReliaPrep™ RNA Tissue Miniprep System
166 kit (Promega, France, Ref. Z6112) following the Promega - ReliaPrep™ RNA Tissue Miniprep System
167 protocol, optimised for RNA extractions of grapevine cell cultures (supplementary protocol available
168 on request from the corresponding author). RNAs underwent DNAse treatment with the Invitrogen™
169 ezDNase™ Enzyme kit to remove genomic DNA contamination from RNA preparations, following the
170 ezDNase™ Enzyme kit protocol. For each sample, quantity and purity were first assessed by measuring
171 the optical density (OD) at 260 nm and 280 nm with a NanoDrop® ND-1000 UV-Vis spectrophotometer
172 (ThermoFisher, USA) and with a 1% (w/v) gel electrophoresis. Then, for each sample, RNA Integrity

173 Number (RIN) was measured using an Agilent 2100 Bioanalyzer System microfluidic on-chip capillary
174 electrophoresis device, following the RNA 6000 Nano kit protocol (Agilent Technologies, Germany).
175 Samples with a RIN > 8.8 were selected and then aliquoted to obtain 20 µL RNA samples at 50 ng.µL^{-1}.
176

177 RNA-seq analyses

178 Selected RNA samples were sent to Novogene Bioinformatics Technology Co., Ltd (Beijing,
179 China) for cDNA library construction and sequencing on the IlluminaNovaSeq 6000 platform (Illumina,
180 San Diego, CA, USA). For each condition, three biological replicates were sent. Raw sequences
181 corresponding to paired-end reads (2 x 150 bp) were treated as described by Althiab-Almasaud *et al*.
182 (2021) and Chirinos *et al*. (2023) but adapted for the grapevine genome. The raw data discussed in this
183 publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are
184 accessible through GEO Series accession number GSE275842
185 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE275842). The reads were aligned on the
186 *Vitis vinifera* L. cv. Chasselas annotated reference genome produced by Djari *et al*. (2024). The quality
187 of these alignments is shown in Supplementary Tables S3A and S3B.
188

189 Differential Expression and downstream analyses

190 First, we normalised the counts according to the transcript lengths to compare transcript
191 expressions in downstream analyses. These normalised expressions can be understood as a relative
192 estimation of transcript counts. The mean value of the biological replicates was calculated, giving as a
193 result the estimated transcript counts for the given condition. Then, Differential Expression (DE)
194 analysis was performed as described by Chirinos *et al*. (2023) with R software version 4.1.1
195 (https://www.r-project.org/) using the DESeq2 package version 1.30.1 with the default relative log
196 expression (RLE) normalisation method, encompassing biases caused by library size and relative size
197 of transcriptomes (Maza *et al*., 2013; Love *et al*., 2014). More precisely, DE analyses were performed
198 as pairwise comparisons between all pairs of conditions (ex: “etha6h_1mM" versus "etha6h_0mM”).
199 The false discovery rate (FDR) was controlled by the Benjamini–Hochberg method with an adjusted p-
200 value (padj) < 0.05. Principal Component Analysis (PCA) was performed on estimated transcript counts
201 with the plotPCA function of DESeq2 after a variance stabilising transformation.
202
203

204 Identification and annotation of the differentially expressed genes (DEGs)

205 The functional annotation of the DEGs was retrieved from the VitExpress platform. When
206 functional annotations were missing, gene ID codes were converted from “Vv” to the taxonomic ID of
207 PNv4 “Vitvi” (Canaguier *et al*., 2017) using the GBFwebtool gene ID converter

208 (https://www.grape.resources.gbfwebtools.fr/converter). The "Vitvi" codes were then used to obtain
209 complementary annotations through MapMan Classification (https://mapman.gabipd.org/home)
210 (Schwacke *et al*., 2019). If both annotations were missing, the "Vv" code was used to extract the
211 corresponding gene sequence using the GBFwebtool "Extract Sequences"
212 (https://www.grape.resources.gbfwebtools.fr/extractSequences) and was subsequently blasted in
213 NCBI to find similar sequences and putative function (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
214

215 Gene Ontology Enrichment analysis

216 Gene ontology (GO) enrichment analysis was performed with R using the enrichGO function
217 from clusterProfiler package version 4.10.1. Specifically, GO analysis was conducted separately on both
218 the up-regulated and down-regulated DEG lists obtained from the comparison between EtOH-treated
219 and control calli. Only DEGs with a |Log2FoldChange|>2 were selected.
220

221 EtOH priming and heat-shock treatments

222 For *Vitis vinifera* cv. Gamay Fréaux cell cultures: 200 µL of liquid growth medium with EtOH at
223 different concentrations (0, 0.1, 1, 10, 100 or 1000 mM) were poured onto three-week-old calli under
224 sterile conditions. Petri dishes were then sealed with micropore surgical tape (3 M, 1.25 cm width,
225 Germany) and returned to the culture chamber for 68 hours. After this incubation time, optimised by
226 preliminary experiments, the heat shock (HS) was applied for 15 minutes in an incubator in the dark,
227 with a peak temperature reaching 47°C (recordings of the heat stress peak can be found in
228 Supplementary Fig. S1.). The Petri dishes were allowed to cool down for 20 minutes before returning
229 to the growth chamber. Controls were treated with a control solution (liquid growth medium without
230 EtOH) and were subjected to heat stress. Double negative controls were treated with a control solution
231 (liquid growth medium without EtOH) and were not subjected to heat stress, but were kept in the dark
232 for the duration of HS.
233 For *Arabidopsis thaliana* HS treatment, the protocol was adapted from Matsui *et al*. (2022)
234 with the following modifications. *Arabidopsis* seedlings grown for 11 days with 0, 0.1 or 1 mM EtOH
235 were washed with deionized water and placed in test tubes containing 1.8 mL of distilled water (15
236 shoots/tube). The heat stress was applied by placing the tubes in a water bath at 43 °C for 30 minutes
237 and then cooling them down in a water bath set at 25 °C for 30 minutes. Control tubes did not undergo
238 HS and were kept in a 25 °C water bath for 1 hour.
239

240 Measurements of cellular leakage after HS treatment

241 To assess the cell damage caused by heat stress in *Vitis vinifera* and *Arabidopsis thaliana*, we
242 selected cellular leakage as a key indicator of cell membrane integrity. Indeed, cellular leakage refers

243 to the uncontrolled release of substances, such as ions, metabolites, or macromolecules, from the
244 inside of a cell to its surrounding environment. This phenomenon typically occurs due to damage or
245 disruption to the cell membrane (Jiang et *al.*, 2015), which normally serves as a selective barrier.
246 For *Vitis* cell cultures, cellular leakage measurements were assessed by measuring the
247 coloured compounds released into the callus rinsing solution. The protocol was adapted from Ilík *et al.*
248 (2018) with the following modifications: 72 hours after HS, calli were harvested, placed in 2 mL
249 Eppendorf tubes, and weighed. To each tube, 1 mL of 100 mM mannitol rinsing solution was added to
250 limit osmotic shocks. The tubes were then vortexed for 15 seconds and incubated at room temperature
251 for 20 minutes on a rotator wheel, followed by centrifugation for 2 minutes at 16,100g. The absorbance
252 of the supernatant was measured at wavelengths of 420 nm, 520 nm and 620 nm (Chervin *et al*., 2001;
253 Lambri *et al*., 2015). The 100 mM mannitol solution was used as a blank.
254 We reported the results as relative absorbances, normalised to the HS controls (absorbance values set
255 to 1) to account for variability between different batches.
256 For *Arabidopsis* seedlings, cellular leakage was assessed by the Index of Injury (Id) (Ilík *et al.*,
257 2018), calculated from electrical conductivity measurements. The Id is a quantitative measure used to
258 assess the extent of damage in plant tissues in response to stress conditions such as temperature
259 extremes. It provides an indication of the degree of cellular or tissue damage, and evaluates the impact
260 of various stressors on plant health. Based on a protocol adapted from Matsui *et al*. (2022), *Arabidopsis*
261 seedlings grown for 11 days were washed with deionized water and placed in test tubes containing 1.8
262 mL of distilled water (15 shoots/tube). The heat stress was applied as described previously. Heat-
263 stressed and control samples were slowly shaken overnight at 30 rpm, after which electrical
264 conductivity was measured with a conductivity metre (Yangers, UK). The samples were then heated
265 for 1h in a 100 °C water bath and shaken at room temperature for several hours, after which the
266 electrical conductivity of the samples was again measured. The index of injury (Id) was calculated based
267 on the protocol described for ion leakage analysis of *Arabidopsis seedlings* by Ilík *et al*. (2018).
268

269 Anthocyanin content determination

270 This protocol was adapted from Vitrac *et al*. (2000), which investigated cell suspensions of *Vitis*
271 *vinifera* (L.) and more notably those of cv. Gamay Fréaux Teinturier. Some modifications have been
272 made: Three-week-old calli were treated with 100 µL of either a 1 mM EtOH or a control solution and
273 were incubated for up to 16 days post-treatment in their growth chamber. Fresh callus tissues were
274 collected, weighed, and placed into 1.5 mL Eppendorf tubes. Each tube received 500 µL of a solvent
275 mixture composed of methanol and 0.32M HCl (85:15, v/v). The tissues were ground with a tube pestle
276 for 1 minute, followed by incubation for 2 hours at 4 °C on a rotator wheel. After incubation, the tubes

277 were centrifuged at 16,000g for 2 minutes. The supernatant was diluted to 1/50th of its original volume
278 with the same solvent.
279 The absorbance of the anthocyanin extract was measured at 535 nm. Anthocyanin content was
280 calculated using the molar extinction coefficient (log ε = 4.53) as reported by Vitrac *et al*. (2000).
281 Methanol and 0.32M HCl (85:15, v/v) solvent was used as a blank.
282

283 Total Polyphenol Index determination

284 The Total Polyphenol Index (TPI) is a measure used to quantify the total amount of
285 polyphenolic compounds present in a sample. The TPI method relies on the absorption of benzene
286 rings at 280 nm, a characteristic feature of polyphenols. This protocol was adapted from Cetó *et al*.
287 (2012) with the following modification: on the same supernatant extracts as those prepared for
288 anthocyanin determination, absorbance was measured at 280 nm using a quartz cuvette. The TPI for
289 each sample was calculated as the absorbance multiplied by the appropriate dilution factor. Methanol
290 and 0.32M HCl (85:15, v/v) solvent was used as a blank.
291

292 Statistical analyses

293 All data in Figures 1 and 4 were analysed by ANOVA and multiple comparison tests, performed
294 with Sigmaplot v.15 (Inpixon, USA). The confidence interval was set at 95% and the p-value is set at
295 0.05. Significantly different groups are indicated by small letters (a, b, c) on the graphs.
296 For RNA-seq data, the statistical analyses were performed using the R software, as specified in the
297 previous paragraphs.
298
299

300 Results

301 Grapevine *Vitis vinifera* L. cv. Gamay Fréaux cell cultures are characterised by 302 hypoxia and physiological ethanol levels in the µM to mM range

303 Figure 1A shows pictures of grapevine cell cultures as a function of development time over five
304 weeks. The increase in callus size throughout their development is noticeable, but between days 28
305 and 35 of development, callus size varies only slightly (average callus weight over 5-week-development
306 can be found in Supplementary Fig. S2). Furthermore, the typical colour of our study model, a bright
307 pink to light red, is evident in the first four weeks of development. A noticeable colour change occurs
308 between the 28th and 35th days of development: by the 35th day, the calli appear duller compared to
309 those grown for 0, 7, 14, 21, and 28 days.
310 We then characterised the levels of oxygen in the grapevine calli during five weeks of
311 development on growth media. Figure 1B shows dissolved O_2 levels at a distance of 2100 µm towards

312 the callus centre as a function of development time. We found that O_2 levels increase substantially and
313 gradually over time of development, starting with concentrations of around 10 µmol.L^{-1} in 7-day-old
314 calli to 150 µmol.L-1 in 35-day-old calli. Dissolved oxygen levels in calli grown for 7 and 14 days were
315 statistically lower than in 35-day-old calli. Complete O_2 profiles inside calli were measured and sections
316 of 14-day-old and 35-day-old calli were observed, and results are shown and detailed in Supplementary
317 Figure S3, and Supplementary Figure S4, respectively. Regarding the natural endogenous levels of
318 ethanol in calli, we found a strong decrease in ethanol concentration as a function of development
319 time, with an inverse relationship to oxygen levels (Fig. 1C). Ethanol concentration was significantly
320 higher in 7-day-old calli than in 35-day-old calli, decreasing from 1726 ± 190 µM to 121 ± 99 µM,
321 respectively.
322 Therefore, we can define the range from 100 µM to 1700 µM as the "physiological" ethanol
323 concentrations present in cell cultures of *Vitis vinifera* L. cv. Gamay Fréaux under normal growth
324 conditions.
325

326 A small exogenous EtOH dose impacts the grapevine transcriptome

327 To investigate the impact of a physiological dose of EtOH on the *Vitis vinifera* transcriptome,
328 we employed RNA sequencing (RNA-seq). Overviewing the transcriptional dynamics of the RNA-seq
329 experiment, the Principal Component Analysis (Fig. 2A) revealed significant distinctions among the
330 generated transcriptomes, with 74% of the variance associated with PC1 and 14% with PC2. The data
331 consistently demonstrates a clustering pattern, wherein transcriptomes align closely according to their
332 respective treatments, forming 4 distinctive clusters (0mM_6h, 1mM_6h, 0mM_24h, and 1mM_24h).
333 This showed that biological variability was smaller than the differences between the four conditions
334 and suggests a discernible impact of a low 1 mM dose of EtOH on the transcriptomes. There is more
335 distance between the control and ethanol treatment at 6 hours than at 24 hours (Fig. 2A). Additionally,
336 it is noteworthy that the 1mM EtOH treatment at 6 hours and at 24 hours show the greatest distance
337 in the PCA plot, whereas the control solution at 6 hours and 24 hours generated the closest
338 transcriptome groups. There were 5527 DEGs in the 6 hours comparison whereas there were 3755
339 DEGs in the 24 hours comparison (Supplementary Fig. S5).
340 To further refine our analysis, we applied more stringent criteria to DEGs: we selected genes
341 with a |Log2FoldChange|>2, in addition to requiring an adjusted p-value < 0.05 (raw results of the
342 Differential Expression Analysis of the comparison 1 vs 0 mM EtOH at 6 hours are available in
343 Supplementary Table S4). For the comparisons at both 6 and 24 hours, the DEG analysis revealed that
344 there was a greater number of down-regulated DEGs (Fig. 2C, D). These results show a transcriptional
345 trend towards inhibition, with most genes under-expressed in ethanol-treated calli. When visually
346 comparing the number of DEGs between the two post-treatment durations, the volcano plot for the

347 6-hour time point shows a greater number of overall DEGs than the plot for the 24-hour comparison
348 (386 vs. 106 genes respectively, see Fig. 2D).
349 These results indicate that even a light change in ethanol significantly impacts the grapevine
350 transcriptome.
351

352 EtOH treatment within the physiological range significantly upregulates small 353 heat shock protein-related gene expressions

354 Considering the higher number of DEGs observed at 6 hours compared to 24 hours post-
355 treatment, we decided to focus on the responses 6 hours after treatment. The complete lists of the
356 386 functionally annotated DEGs is available in Supplementary Table S5, sorted out in two sublists:
357 Supplementary Table S5A provides the down-regulated DEGs (254 genes) and Supplementary
358 Table S5B provides up-regulated DEGs (132 genes). Figure 3A shows the results of this GO enrichment
359 for the up-regulated DEGs (p-adj < 0.05 and a Log2FoldChange > 2) while Figure 3B shows the GO
360 enrichment for the down-regulated DEGs (p-adj < 0.05 and a Log2FoldChange < - 2) (the overall GO
361 analysis results can be found in Supplementary Tables S6 and S7 respectively).
362 We found that genes annotated as “response to heat” (GO:0009408) and “response to temperature
363 stimulus” (GO:0009266), “protein self-association” (GO:0043621) and "unfolded protein binding"
364 (GO:0051082) were the most represented with a gene ratio of 0.158, among the 132 up-regulated
365 DEGs (Figure 3A). For down-regulated genes, we found that genes annotated as “secondary
366 metabolism process” (GO:0019748) and “secondary metabolite biosynthetic process” (GO:0044550)
367 were the most represented among the 254 down-regulated DEGs, with gene ratios of 0.08 and 0.07
368 respectively (Fig. 3B).
369 Given these results, we had to examine the specific genes comprising the enriched families in
370 both DEGs lists. First, we examined the specific genes comprising the up-regulated enriched family
371 associated with the GO term "Response to heat". This list includes 15 DEGs, presented in Figure 3C
372 (their respective gene IDs, functional annotations, Log2FoldChange values, and adjusted p-values can
373 be found in Supplementary Table S8). When looking at the functional annotations in Figure 3C, all the
374 up-regulated DEGs are small Heat Shock Proteins (sHSP) and they are sorted out in two major groups:
375 9 genes are cytosolic class I sHSP and 5 genes are cytosolic class II sHSP. The last gene is a class-P sHSP.
376 The Log2FoldChanges of these DEGs range from 2.09 to 7.09 and their associated p-adj values are very
377 low (0.039 to 5.19^{-12}), providing a high level of confidence that the ethanol treatment is responsible
378 for this differential expression.
379 Then, we examined the specific genes comprising the down-regulated enriched family associated with
380 the GO term "Secondary metabolism process". This list includes 16 DEGs, presented in Supplementary
381 Figure S6 (their respective gene IDs, functional annotations, Log2FoldChange values, and adjusted p-
382 values can be found in Supplementary Table S9). When looking at the functional annotations in

383 Supplementary Figure S6 we can notice genes involved in the general phenylpropanoid pathway like
384 the pivotal phenylalanine ammonia-lyase (PAL), and genes involved in phenylpropanoid down-stream
385 pathways. Indeed, genes involved in pathways for stilbenoid and flavonoid biosynthesis, such as
386 stilbene synthase (STS) and chalcone synthase (CHS) are down-regulated. The Log2FoldChanges
387 associated with these DEGs range from -2.02 to 4.90, being less extremes than the ones associated
388 with the "Response to heat" genes.
389

390 ## Ethanol-priming reduced heat-induced cell leakage

391 Following the observation of significant and highly up-regulated sHSPs revealed in the GO
392 analysis, we looked for thermotolerance phenotypes in ethanol-primed calli after heat stress.
393 The results are detailed in Figure 4. We observed that the double negative controls, which entails
394 neither ethanol priming nor exposure to heat stress, exhibited the lowest absorbances at 420, 520 and
395 620 nm respectively (cyan bars, Fig. 4A-C), showing the basal cellular leakage in our experimental
396 conditions. In contrast, cellular cultures exposed to both the control pre-treatment (0 mM EtOH) and
397 HS demonstrated the highest absorbance values at all three wavelengths. Calli primed with EtOH
398 solutions ranging from 0.1 to 10 mM, displayed a continuous decreasing trend in the absorbance values
399 measured at 420 nm, 520 nm, and 620 nm, compared to the controls. The absorbances increased again
400 within calli primed with 100 and 1000 mM EtOH solutions. These particular inverted bell-shaped curves
401 are observed across all three monitored absorbances.
402 Overall, calli primed with concentrations of 1 and 10 mM showed strong reductions in
403 absorbance around 35-45% compared with the HS-control. These results show that priming grapevine
404 cellular cultures with low physiological doses of ethanol (i.e. 0.1 to 10 mM) reduced the cellular
405 compound leakage after exposure to a mild heat shock. Moreover, a complementary experiment was
406 conducted on *Arabidopsis thaliana* seedlings to confirm that low doses of EtOH could limit cellular
407 leakage in another plant's tissues and in a different plant species. Figure 4D presents ion leakage for
408 *Arabidopsis thaliana* seedlings. We found that in control seedlings, the injury index at 83% was
409 significantly higher than in seedlings treated with 0.1 and 1 mM EtOH, which were at 69% and 75%
410 respectively. This corresponds to a significant reduction in the injury index by 14% for the 0.1 mM
411 ethanol treatment and 8% for the 1 mM ethanol treatment compared to control. This result shows
412 that priming *Arabidopsis* seedlings with low physiological doses of ethanol (i.e. 0.1 and 1 mM)
413 significantly reduced the ions released after exposure to a mild heat shock, and further reinforces the
414 results described for the Gamay cell cultures.
415

416 ## Ethanol-priming does not impact polyphenol content in grapevine calli

417 As shown in Figure 3B, the GO terms 'Secondary metabolic process' and 'secondary metabolite
418 biosynthetic process' are significantly enriched among the 254 down-regulated DEGs, many of which
419 are linked to polyphenol synthesis (see Supplementary Fig. S6). Given this significant down-regulation
420 observed in secondary metabolism, and particularly the down-regulated PAL and CHS transcripts, we
421 investigated polyphenol content in ethanol-primed calli. To assess this further, we monitored
422 anthocyanin and total polyphenol content as a function of time in grapevine cell cultures, following an
423 initial 1 mM EtOH treatment. The supplementary Figure S7 illustrates the changes in anthocyanin
424 concentration and total polyphenol index in cell extracts. Both graphs show an increasing trend as a
425 function of time, however, there was no significant difference in anthocyanin concentrations
426 (Supplementary Fig. S7A) or in the total polyphenol index (Supplementary Fig. S7B) between the
427 ethanol-primed and control treatments.
428
429

430 **Discussion**

431 Grapevine cell cultures, a model experiencing hypoxia and ethanol accumulation

432 A recent study by Xiao *et al (*2018) showed low levels of oxygen in grape berries and even
433 anoxia in the centre of the berries. Our grapevine cell culture model shows the same trend, with
434 developmental hypoxic to anoxic oxygen levels and inversely correlated ethanol concentrations in the
435 calli, especially when they undergo rapid growth in the first 2 weeks after subculturing (Fig. 1B,C). This
436 rapid growth is associated with a high rate of cell division and high cellular oxygen consumption in
437 most plant tissues (Raymond *et al*., 1995). The increase in oxygen levels in calli, as they age, is probably
438 linked to a decrease in their growth and metabolic rates. This slower growth allows oxygen to diffuse
439 faster than it is consumed by the cells. Additionally, after five weeks of growth, cross-sections
440 (Supplementary Fig. S4) revealed a heterogeneous structure with invaginations and gaseous interstices
441 within the callus, further facilitating air and oxygen diffusion. A close relationship exists between
442 oxygen and ethanol levels in plants, in which decreased oxygen levels result in increased ethanol
443 concentrations, due to the shift from aerobic to anaerobic respiration (Bui *et al*., 2019). Thus, as
444 dissolved oxygen levels increase over callus developmental time, it is logical to observe a
445 corresponding decrease in ethanol levels. Therefore, we can conclude that our cell culture model
446 naturally experiences developmental hypoxia, leading to ethanol accumulation. This validates the cell
447 culture model for studying the impact of small changes in ethanol concentrations. These latter could
448 be related to climate change, as rising temperatures in this context further reduce dissolved oxygen
449 levels in water and organisms, while increasing respiration rates. These changes lead to hypoxic
450 metabolism in certain organisms and tissues, and to ethanol production. The question arises whether

451 ethanol serves as a signal prompting organisms to adapt, or if it is simply an end-product of hypoxic
452 metabolism (Diot *et al*., 2024).
453

454 Small exogenous application of EtOH triggers a fast response in the grapevine 455 transcriptome and cells seem to perceive it as a stress.

456 We present evidence that small changes in ethanol concentration generate quick responses at
457 the transcriptomic level in Gamay cell cultures. On the PCA plot (Fig. 2A), the closely grouped
458 transcriptomes based on their respective treatments and the high explained variance demonstrate the
459 significant impact of ethanol on gene transcripts. The sum of the variances (PC1 + PC2), which accounts
460 for 88% of the total variance, indicates that the two-dimensional plot effectively represents the impact
461 of the ethanol treatment and the two sampling time points (6 and 24 hours). It is possible that the
462 highly controlled *in vitro* culture environment and the use of identical cloned cells limit the biological
463 variability in our experimental design. The fact that Gamay cell cultures can sense very small changes
464 in ethanol concentration (1 mM) is evidenced by the 386 DEGs identified at 6 hours (Fig. 2D),
465 suggesting that a small amount of ethanol is quickly detected by the grapevine cells and prompts a
466 strong and immediate transcriptional response. The reduction in the number of DEGs to 106 at 24
467 hours post-treatment (Fig. 2D) shows that the transcriptional response diminished over time. This
468 result is further reinforced by the decreased number of DEGs with a high Log2FoldChange after 24
469 hours compared to 6 hours (Fig. 2C versus Fig. 2B). This trend suggests that the transcriptional
470 response to low doses of ethanol is not only strong and immediate but also relatively transient. The
471 general trend of downregulation of the transcriptomic dynamics when comparing 1 mM EtOH to
472 control treatments (Fig. 2D), at 6 and 24 hours post-treatment, is often observed in plant responses to
473 stresses. Indeed, Ul Haq *et al.* (2019) showed that a plant's common strategy to cope with stressful
474 situations is reducing the synthesis of normal proteins, while enhancing the production of stress-
475 related proteins, such as HSPs. This trade-off is clearly illustrated at the transcriptomic level in our
476 study: Gamay calli downregulate genes involved in secondary metabolism (Fig. 3B) while
477 simultaneously overexpressing a set of genes related to stress proteins following the addition of
478 exogenous ethanol (Fig. 3A). This GO analysis revealed that cell sHSP genes were upregulated in calli
479 treated with 1 mM EtOH compared to the control at 6 hours (Fig. 3A). sHSPs function as early
480 responders to assist cells in managing proteins destabilized by stress conditions (Janowska *et al*., 2019),
481 and their induction represents a rapid and intense emergency response (Parsell and Lindquist, 1993).
482 This finding aligns with our RNA-seq data, as we observed that, just 6 hours after treatment with 1 mM
483 EtOH, some sHSP genes were expressed more than 64 times than in the control (Fig. 3C). Our study
484 suggests that a minor change in ethanol is sensed by the cells as a stress, prompting them to initiate
485 adaptive responses.

486

487 Responses of grapevine cells to physiological doses of ethanol: upregulation of
488 small heat shock protein transcripts.

489 Our results show that the genes associated with the GO terms "Response to heat" and
490 "Response to temperature stimulus" are the most statistically overrepresented 6 hours after a 1mM
491 EtOH treatment, notably the sHSPs (Fig. 3C).
492 Firstly, the ability of ethanol at physiological doses to stimulate sHSP synthesis has already been
493 observed in the animal kingdom. Indeed, some studies showed that ethanol at 50 mM induced sHSP
494 genes in mouse embryonic neural stem cells (Choi *et al*., 2011) and EtOH at 60 mM increased the levels
495 of HSP27 in mouse cortical neurons (Pignataro *et al*., 2007).
496 sHSPs: HSPs are classified into five major subfamilies based on their molecular weights:
497 Hsp100, Hsp90, Hsp70, Hsp60, and small heat shock proteins (sHSPs) (Sun and MacRae, 2005). These
498 latter are differentiated from the other HSPs by their smaller molecular weight -typically, plant sHSPs
499 range between 15 and 30 kDa-, and are the most abundant in plants (Vierling, 1991). The sHSPs are
500 expressed during the normal developmental process of plants (Lindquist and Craig, 1988), and are
501 known for their response to a wide range of environmental stresses, including heat, cold, drought,
502 salinity, and oxidative stress; and it seems that the high diversification of plant sHSPs reflects a
503 molecular adaptation to stress conditions. As it is clear that sHSPs have evolved to be sensitive to small
504 changes in pH or in metal ions near physiological concentrations (Janowska *et al*., 2019), it would be
505 logical that they are also sensitive to other small changes near-physiological state like, in our case,
506 small changes in ethanol concentration. The small HSPs are particularly renowned for their crucial role
507 in maintaining proteostasis when expressed in response to cellular stress in plant systems (Haslbeck
508 and Vierling, 2015). Indeed, they specialized in preventing protein aggregation by binding to partially
509 unfolded proteins under stress conditions (McLoughlin *et al*, 2016; Janowska *et al*., 2019; Lee *et al*.,
510 1995, 1997), differentiating from other larger HSPs in their role, as sHSPs do not typically refold
511 proteins. Besides this activity, sHSPs can function as membrane stabilisers and ROS scavengers or act
512 synergistically with antioxidant systems. Overall, they play a key role in maintaining membrane fluidity
513 and permeability under some stress (Aghdam *et al*., 2013). However, ethanol was shown to modify the
514 fluidity of phospholipid bilayers (Patra *et al*., 2006), possibly creating a link between ethanol,
515 membrane organization, and the induction of sHSPs.
516 Class CI and CII sHSPs: In Figure 3C, the key genes attracting our attention were sHSP genes
517 encoding for class CI and CII subfamilies. These are the two most abundant classes of plant cytosolic-
518 localised sHSPs. CI sHSPs typically comprise the largest family of sHSP genes in higher plants (Waters,
519 2013) while the CII gene family is typically smaller. Basha *et al*. (2010) examined the structural and
520 functional differences between the CI and CII subfamilies. They reported that both the CI and CII

521 subfamilies have chaperone activities and they were effective at preventing irreversible aggregation,
522 illustrating their role in the prevention of future damages made to the cell. Therefore, it is not so
523 surprising that 6 hours after the EtOH treatment, we observed a significant up-regulation of these
524 protein transcripts. Thus, we can hypothesize that the plant may use very small changes in ethanol to
525 prevent further damage due to stresses. Indeed, ethanol has been shown to induce sHSP in soybean
526 (Kuo *et al*., 2000) and in rice (Guan *et al.*, 2004), even if the ethanol doses tested were 5% (v/v)
527 corresponding to a concentration of nearly 1M, which can be considered like a "hammer" dose.
528

529 Small changes in ethanol concentration confer thermotolerance to grapevine 530 calli by reducing cellular leakage, a trend confirmed by experiments with 531 *Arabidopsis* seedlings

532 Given that our plant model is a culture of cells rich in anthocyanins (Guan *et al*., 2016; Kong *et*
533 *al*., 2021), we drew inspiration from studies demonstrating the release of pigments following heat
534 stress (Lambri *et al*., 2015). Zhang *et al*. (2005) showed that heat treatment (38 °C for 10 h) induced
535 injuries in the ultrastructure of *Vitis* mesophyll cells, then linked to increase in conductivity associated
536 with membrane leakage, and also linked to malondialdehyde increase. In oenology, the postharvest
537 heat treatment of grape berries has been known for a long time to increase berry skin cell damage and
538 facilitate the release of anthocyanins (Lambri *et al*., 2015). These latter authors showed that heating
539 grape berries at various temperatures from 60 to 80°C for various times 3 to 60 min, increased the
540 absorbances at the main three wavelengths defining red wine colour intensity: 420, 520 and 620 nm,
541 the main red pigments being anthocyanins (Lambri *et al*., 2015). In our study, after preliminary
542 experiments, we chose a rather mild heat treatment (Supplementary Fig. S1) in order to illustrate
543 differences induced by ethanol priming. In our Figure 4A, 4B and 4C, significant differences in pigment
544 leakage indicate that ethanol priming protected grapevine calli from damage due to exposure to heat
545 stress, compared to control ("0"). This protective effect was also shown in a different plant species.
546 Indeed, significant differences in electrical conductivity indicate that ethanol priming also protected
547 *Arabidopsis* seedlings from damage due to exposure to heat stress (Fig. 4D), compared to control ("0").
548 In other words, ethanol priming effectively reduced cell leakage caused by heat treatment,
549 demonstrating that a low dose of ethanol enhances tolerance to heat stress, and suggesting that the
550 ethanol-induced-sHSPs are related to the acquisition of thermotolerance. Increasing data suggest a
551 strong correlation between sHSP accumulation and plant tolerance to stress (Liu *et al*., 2012; Wang *et*
552 *al*., 2004), like our data. These latter authors suggest that the protective effect involves membrane
553 stabilization. According to Ilík *et al.* (2018), heat acclimation is connected to an increase in the degree
554 of saturation of fatty acids of membrane lipids, which in turn leads to increased rigidity of cell
555 membranes and increase in their thermostability. A search for desaturase-related-genes in our RNA-
556 seq data did not bring evidence that ethanol priming is regulating this type of enzyme, at 6 or 24 hours

557 post-treatment. Nevertheless, Tsvetkova *et al*. (2002) showed that association between sHSPs and
558 membranes may constitute a general mechanism that preserves membrane integrity during thermal
559 fluctuations.

560 Plants have evolved many mechanisms for adaptation to dynamic and adverse environmental
561 conditions. In addition, they have acquired a form of 'stress memory' in that they retain their response
562 to an initial stress, which primes their response to a second stress, allowing them to respond more
563 rapidly and strongly (Savvides *et al*., 2016). One adaptation is their ability to produce a variety of
564 compounds that induce the expression of stress-related genes, whose encoded products protect
565 cellular components from the adverse impact of abiotic stress (Sako *et al*., 2020). This phenomenon,
566 called chemical priming, is evident in our results, as cell cultures that underwent ethanol priming
567 showed better protection against mild heat stress. Todaka *et al*. (2024) demonstrated that a 20 mM
568 ethanol treatment enhanced heat stress tolerance in tomatoes, and highlighted the potential of
569 ethanol-based chemical priming as a technology to mitigate heat stress damage in crops. Crosstalk
570 between heat and ethanol stresses, and heat and ethanol tolerance, has already been shown in several
571 species and seems to be a conserved trait across kingdoms. In plants, this has been shown by Matsui
572 *et al*. (2022). And in other kingdoms, HSP induction correlates with induced tolerance to high
573 concentrations of ethanol: in mammals, Chinese hamster (Li, 1983); in microorganisms,
574 *Saccharomyces cerevisiae* (Plesset *et a*l., 1982).

575 One point left to discuss is the timeframe of the responses. The sHSPs expression is often part
576 of an immediate stress response (Hu *et al*., 2022) but their synthesis is not maintained through time
577 (Altschuler and Mascarenhas, 1982). Our results reflect this, as no sHSP genes were up-regulated 24
578 hours post-ethanol treatment when compared to control. However, 68 hours after ethanol priming,
579 thermotolerance phenotypes were observed (Fig. 4A-C). Vierling (1991) reported that pea plants
580 exposed to heat stress accumulated chloroplastic sHSPs and class CI HSPs, even after the stress had
581 ended. The half-life of these proteins was found to be around 52 hours for chloroplastic sHSPs and 37
582 hours for cytoplasmic HSPs. This shows that although the expression of small heat shock proteins is
583 transient, the sHSPs themselves are quite stable. The persistence of these HSPs suggests that their
584 participation in recovery processes could be as important as their possible role during the stress period
585 itself (Vierling, 1991). However, the authors of the present study have to report that priming is
586 effective in mitigating heat stress when the stress is mild. Indeed, when the stress exceeds a certain
587 threshold (higher than sublethal heat shock), the priming effect diminishes, and the treated cells and
588 *Arabidopsis* seedlings exhibit worse leakage compared to the control (data not shown).
589
590

591 ## Responses of grapevine cells to physiological doses of ethanol: downregulation
592 of key polyphenol-related transcripts, without effects on anthocyanin and
593 polyphenol contents

594 As previously discussed, the general trend in the downregulation of secondary metabolism is
595 a classical plant response to stresses (Ul Haq *et al.*, 2019; Bilgin *et al*., 2010; Liu *et al*., 2012). However,
596 no significant difference was observed between EtOH treated and untreated calli regarding the
597 content of anthocyanins and the content of total polyphenols (Supplementary Fig. S7). We have two
598 main hypotheses to explain the difference between the transcriptomic and the physiological levels.
599 First, it could be that because the anthocyanin and polyphenol contents are so important in Gamay
600 Fréaux calli, that the transient downregulation of the enzymes involved in the biosynthesis of
601 polyphenols results in a too small physiological change. Indeed, the Gamay Fréaux is a teinturier grape
602 and stands out among grape cultivars due to its rare and unique red-fleshed berries as well as red skin,
603 which is uncommon compared to the majority of grape cultivars having red skin colour but white-
604 coloured flesh (Guan *et al*., 2016). Second, the polyphenol pathway is complex with numerous proteins
605 involved. The fact that the expression of some of these protein transcripts is inhibited does not ensure
606 that polyphenolic compounds accumulate at different levels.

607

608 ## Plant response to ethanol displays hormesis characteristics and ethanol could be
609 a potential messenger of hypoxia and other stressful conditions.

610 Because we saw the sHSPs subfamily being extremely overexpressed in the RNA-seq
611 experiment and in order to examine a physiological response of grapevine cell cultures to low doses of
612 ethanol, we assessed the cell damages resulting from a heat shock in calli primed with ethanol at
613 different concentrations. The results (Fig. 4A-C) show a clear biphasic dose-response curve for the
614 absorbances tested, typical of an hormesis effect. Hormesis is a phenomenon observed in biology
615 where exposure to low doses of a stressor or toxin results in a beneficial effect on an organism,
616 contrary to the expected harmful effect at higher doses (Calabrese and Baldwin, 2002; Calabrese and
617 Baldwin, 2003). This concept challenges the traditional linear dose-response model by proposing U-
618 shaped or inverse U-shaped response curves (Erofeeva, 2022). According to Calabrese and Baldwin
619 (2002; 2003), hormesis is considered an adaptive response of organisms to stimuli (environment,
620 chemicals, …). At low doses, the stressor triggers adaptive responses within cells and organisms, such
621 as increased antioxidant production (Wang *et al*., 2023) and repair mechanisms (Scott, 2008), which
622 enhance resilience and improve overall health. Calabrese and Baldwin (2002; 2003) suggest that this
623 stimulatory response could be the result of compensatory biological processes, following an initial
624 disruption in homeostasis, i. e. in our study, the slight change in ethanol concentration. Although there
625 is no single biological mechanism that can explain hormesis across plants, Agathokleous *et al*. (2020)

626 suggest that certain general principles regarding the mechanisms of hormesis across different species
627 and stress-inducing agents can be identified. These authors note that low doses of stress induce a mild
628 increase in the production of reactive chemical species, hormones, and enzymatic and nonenzymatic
629 antioxidants, as well as upregulation of proteins (e.g., heat shock proteins); and that these responses
630 lead to reduced toxicity if plants are subsequently exposed to severe stress, within a preconditioning
631 framework. Our study shows both an increase in heat shock proteins transcripts (Fig. 3B) and reduced
632 HS toxicity (Fig. 4) after an ethanol treatment. This hormesis effect of ethanol has already been seen
633 in a study by Middleton *et al* (1978) on its impact on the architecture of pea roots, where the mean
634 root length was increased by an ethanol concentration as low as 0.002 $ml.L^{-1}$ (equivalent to 0.0002%
635 or 0.034 mM). Moreover, this positive impact of low doses of ethanol has been observed and
636 confirmed by more recent studies. Indeed, Bhattacharya *et al*. (1985) showed that ethanol increased
637 the root formation in mung bean, with a maximum reached at 0.2% (equivalent to 34.25 mM), and Wu
638 *et a*l. (2019) reported that ethanol improved both biomass (at 0.0125 - 0.05 $mL.L^{-1}$, equivalent to 0.21
639 - 0.86 mM) and micronutrient accumulation in oilseed rape. The characteristic response of hormesis
640 can be observed in the results of transpiration of Gamay cuttings subjected to water stress and EtOH
641 priming, in a companion article published in this issue (Ait Kaci *et al*., 2024). And finally, the curve
642 shapes of cellular leakage observed in our study (Fig. 4), on two different plant species, strengthen the
643 previous results. Thus, our results suggest that ethanol at physiological concentrations interacts with
644 grapevine and *Arabidopsis* biological systems in a complex manner, where small amounts can activate
645 adaptive and beneficial responses, while larger amounts overwhelm these systems, leading to toxicity.
646 Given its potential role in activating adaptive responses, we hypothesise that ethanol may serve as a
647 signalling molecule in plants and could be a potential messenger of stressful conditions.
648 Further research is needed to explore ethanol-induced hormesis in plants, specifically to
649 elucidate how stimulation and inhibition vary with ethanol concentration. While the inhibition
650 observed at high concentrations is expected as it aligns with the onset of toxicity, the stimulation effect
651 at low concentrations warrants more detailed investigation. Considering ethanol status as a small
652 molecule with significant diffusive capabilities, notably through membranes, and considering its
653 hydrophobic nature, the manner by which cells perceive small variation in ethanol concentration is still
654 a matter of research and debate (Diot *et al*., 2024).
655
656

657 ## Conclusion

658 Our study demonstrated that grapevine cells sense ethanol at a physiological concentration,
659 inducing a strong and rapid transcriptomic response, typical of stressors. This transcriptomic response
660 had physiological repercussions, as evidenced by the thermotolerance biphasic dose-response curves,

661 suggesting that ethanol could act as a potential molecular messenger in stressful situations in plant
662 systems, rather than just being an end-product metabolite of hypoxic metabolisms. Harnessing this
663 response could be a promising strategy to prepare plants for more challenging stress conditions.
664 Indeed, plants biosynthesize various compounds, such as phytohormones and other metabolites, to
665 adapt to adverse environments. Recent studies have demonstrated that exogenous treatment of
666 plants with chemical compounds can enhance abiotic stress tolerance by inducing molecular and
667 physiological defence mechanisms, a process known as chemical priming (Sako *et al*., 2020). The
668 development of effective strategies that mitigate abiotic stress is essential for sustainable agriculture
669 and food security, especially with continuing global population growth. Chemical priming is believed
670 to represent a promising strategy for mitigating abiotic stress in crop plants. Given its low cost and
671 environmental non-persistence, we propose that ethanol could be a good candidate for plant chemical
672 priming and a valuable tool in agriculture, especially for mitigating environmental stress impacts on
673 crops.

674

675

676 ## Supplementary data

677 The following supplementary data are available at the bottom of this file or can be requested from the
678 corresponding author, when dealing with Excel files.

679

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681 **Fig. S1.** Heat-stress monitoring in Gamay cell cultures.

682 **Fig. S2.** Average weight of a callus of Vitis vinifera cv. Gamay as a function of development time
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687 **Fig. S5.** Transcriptional dynamics showing the number of DEGs upregulated and downregulated, 6
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689 **Fig. S6.** Details of the 16 DEGs within the enriched GO term "Secondary metabolism process".

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692

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698 **Table S4.** Results of the Differential Expression Analysis of the comparison 0 vs 1 mM EtOH at 6h

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701 **Table S6.** Overall GO enrichment analysis results for the up-regulated DEG list (132 genes).

702 **Table S7.** Overall GO enrichment analysis results for the down-regulated DEG list (254 genes).

703 **Table S8.** Details of the up-regulated DEGs within the enriched GO term "Response to heat",
704 resulting from the GO enrichment analysis.

705 **Table S9.** Details of the down-regulated DEGs within the enriched GO term "Secondary metabolism
706 process", resulting from the GO enrichment analysis.

707

708

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717

718 ## Author contributions

719 C.C. and A.Diot conceptualised the project and designed the research. A.Diot performed the
720 experimentations. G.M. and A.Djari performed the RNA-seq data cleaning, reads alignment and quality
721 assessment of the reads. A.Diot, E.M. and G.M. performed the analyses on R and O.D.V., G.M. and
722 A.Diot made the R graphs. A.Diot and C.C. wrote the original manuscript draft, and all co-authors
723 contributed to editing the manuscript. A.Diot, S.B. and C.C. were involved in funding acquisition, C.C.
724 and S.B. were involved in A.Diot's PhD supervision.

725

726 ## Conflict of interest

727 The authors declare they have no conflict of interest.

728

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Data availability

The data underlying this article will be shared upon request to the corresponding author.

Companion article

This article is one of two companion articles submitted to the same journal. The second article by Ait Kaci et al. is entitled “Ethanol reduces grapevine water consumption by limiting transpiration”.

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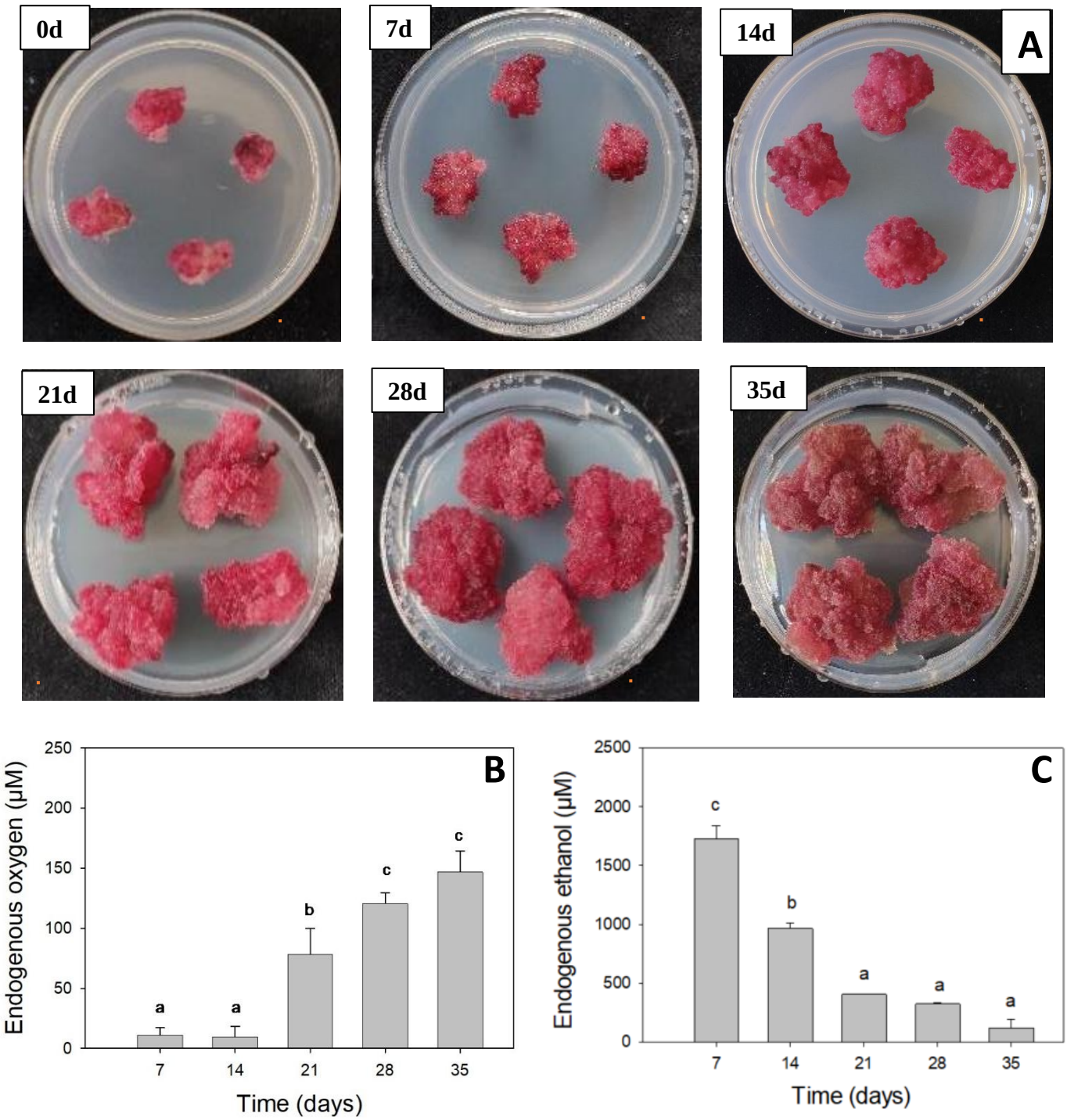


Fig. 1. A) Photographs of Gamay calli at 0 day, 7 days, 14 days, 21 days, 28 days, and 35 days after subculturing onto Petri dishes. The calluses selected are representative of the general development observed. Scale: orange bar represents 1 cm. **B)** Dissolved O_2 levels in callus at a distance of 2100 µm towards the callus centre as a function of development time (0, 7, 14, 21, 28 and 35 days), n = 4 biological replicates. **C)** Ethanol concentration as a function of development time (0, 7, 14, 21, 28 and 35 days), n = 3 biological replicates. Error bars represent standard error and different small letters indicate a significant difference at $p < 0.05$ by multiple comparisons using Fisher's LSD for endogenous oxygen content and Tukey's HSD for endogenous ethanol content.

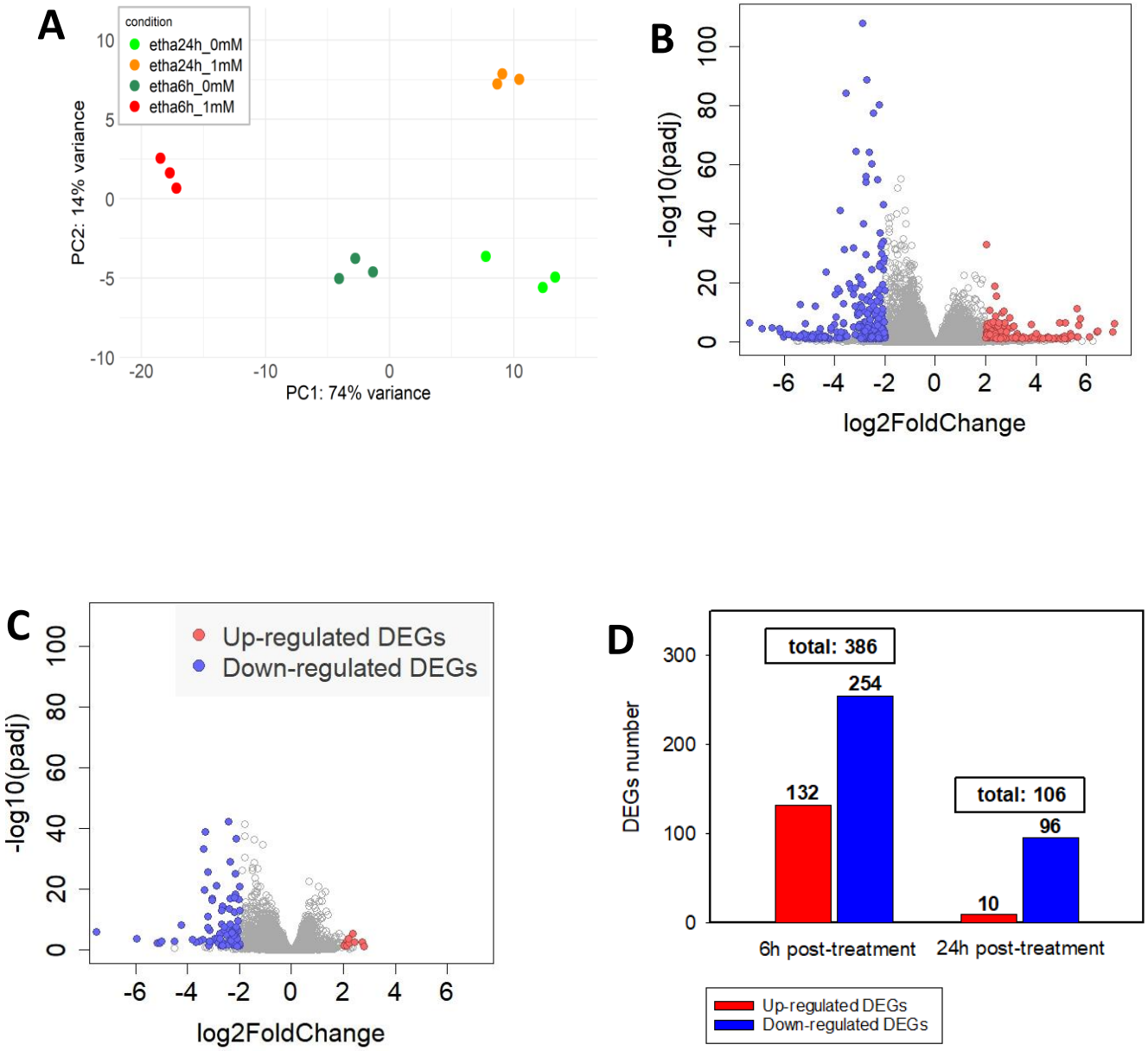


Fig. 2. **A)** Principal Analysis Component of transcriptomic profiles of Gamay Fréaux grape cell cultures 6 and 24 hours after a control 0 mM (green and dark green) or 1 mM (orange and red) ethanol treatment. Every symbol represents one replicate. **B)** Volcano plot of 24655 genes for differential gene expression, at 6 hours post-treatment. **C)** Volcano plot of 24655 genes for differential gene expression, at 24 hours post-treatment. For both Volcano plots: cell cultures treated with 1mM EtOH versus control cell cultures. Scattered points represent genes: the x-axis is the Log2FoldChange for the comparison of EtOH treated cells vs control cells, whereas the y-axis is the negative log10 of adjusted p-values (where P is the probability that a gene has statistical significance in its differential expression). Differentially Expressed Genes (DEGs) that are significantly upregulated (Log2FoldChange > 2 and adjusted p-value < 0.05) are marked in red, while significantly downregulated genes (Log2FoldChange < -2 and adjusted p-value < 0.05) are marked in blue. Genes that do not meet both the significance threshold and one of the log2FoldChange cutoffs are shown in grey. **D)** Comparative Analysis of DEG number for the comparison 1mM EtOH versus control, at two time points. The DEGs shown in the figure are any genes with a p-adj < 0.05 and a Log2FoldChange > 2 for the up-regulated DEGs or Log2FoldChange < -2 for the down-regulated DEGs.

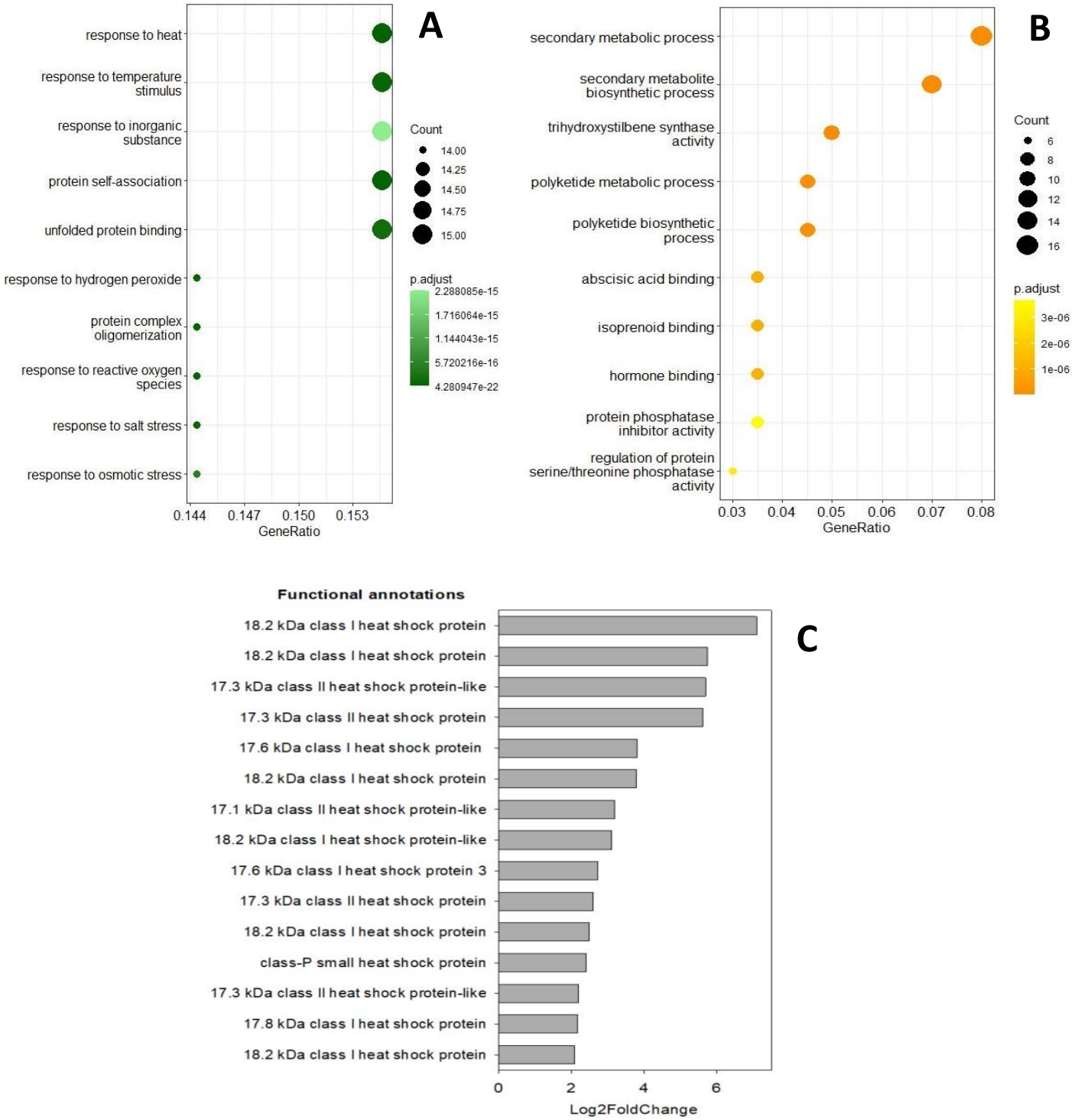


Fig. 3. Dot plots of the Gene Ontology (GO) enrichment analysis of **A)** up-regulated DEGs and **B)** down-regulated DEGs for the comparison of 1mM EtOH versus 0mM at 6 hours post-treatment. DEGs used for the GO enrichment analysis are genes with p-adj < 0.05 and cutoffs as follows: Up-regulated DEGs–with a Log2FoldChange > 2 and down-regulated DEGs with a Log2FoldChange < -2. The size of the dots represents the number of genes in the significant DE gene list associated with the GO term and the colour of the dots represent the enrichment P-adjusted value. **C)** Details of the 15 up-regulated DEGs within the enriched GO class of "Response to heat "and ordered by their respective Log2 Fold Changes for a 1 mM ethanol treatment compared to control, at 6 hours post-treatment.

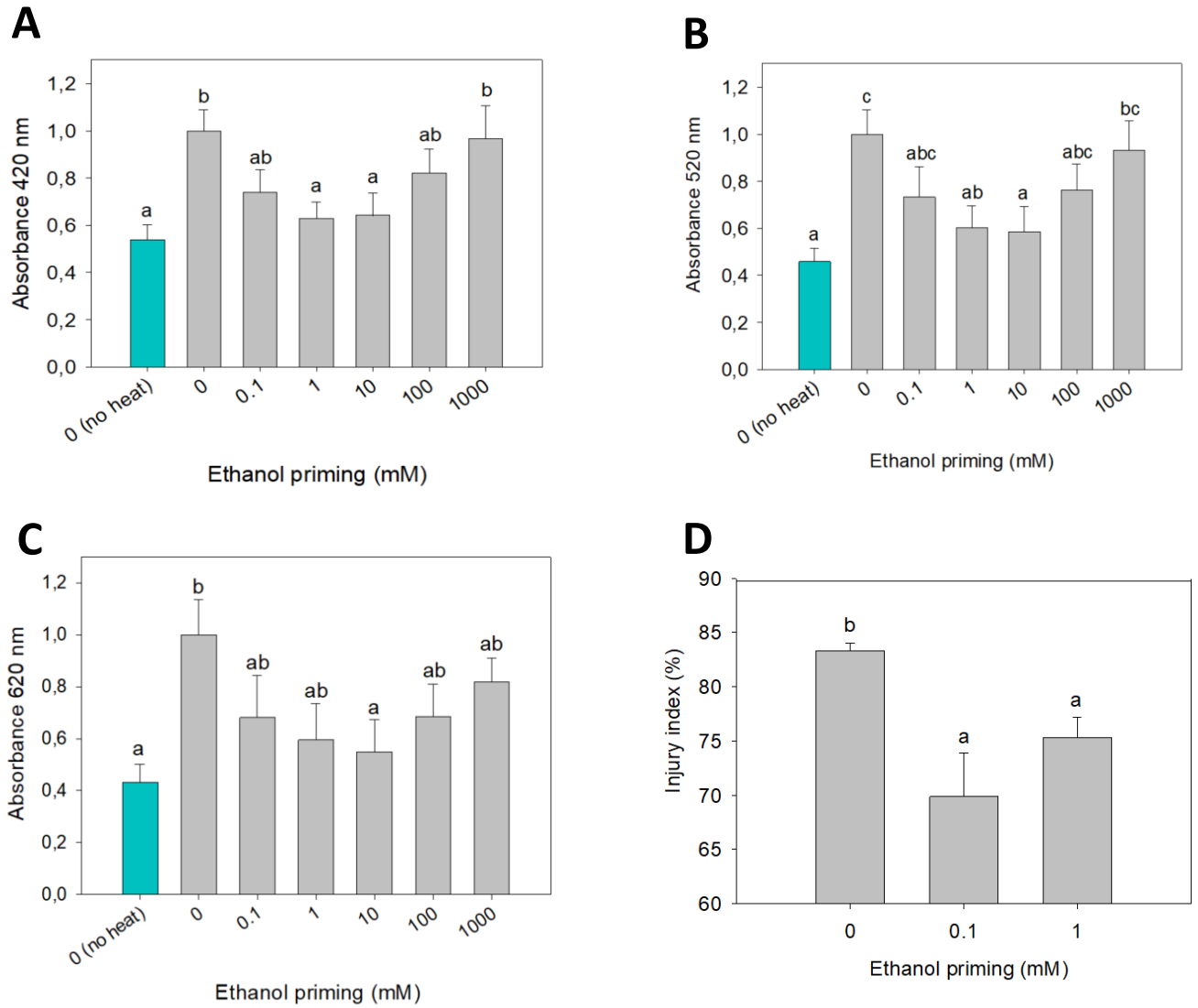
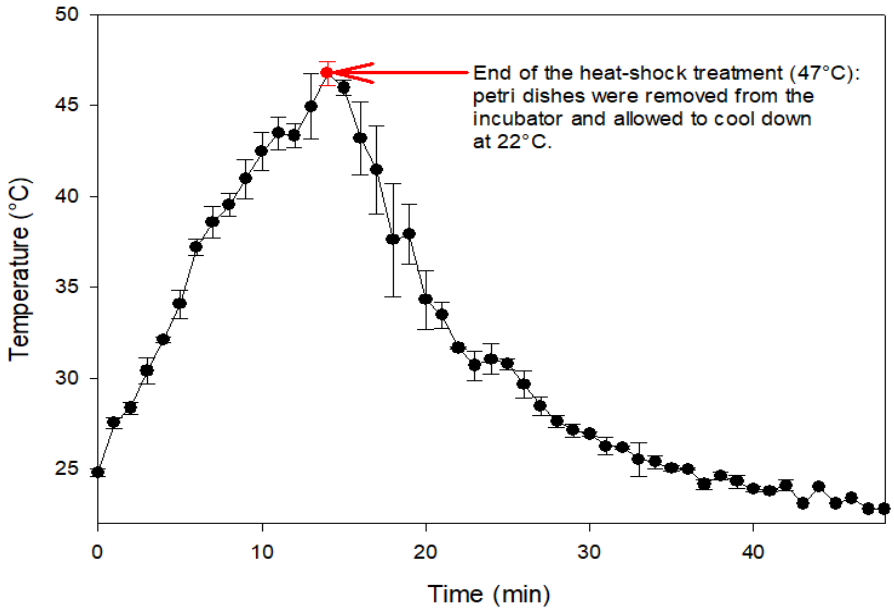
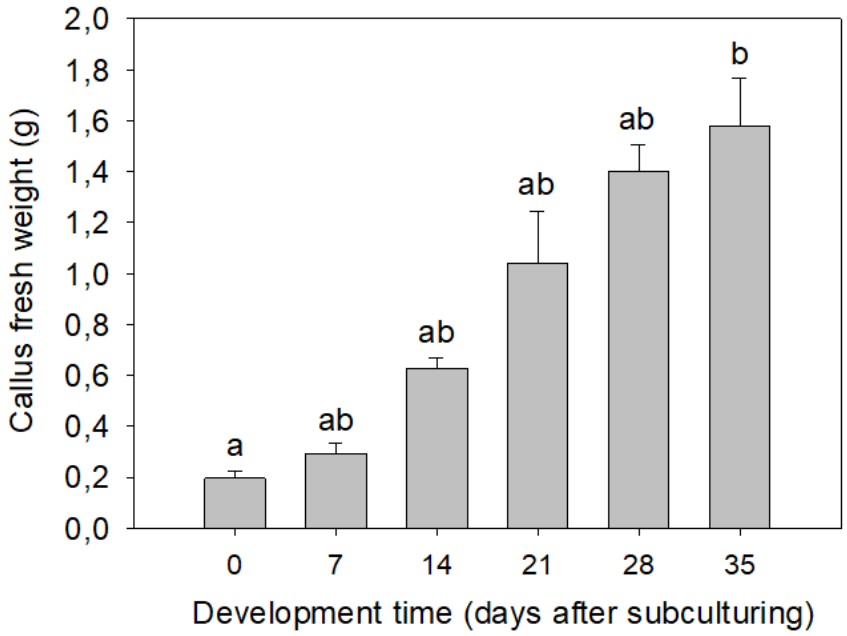


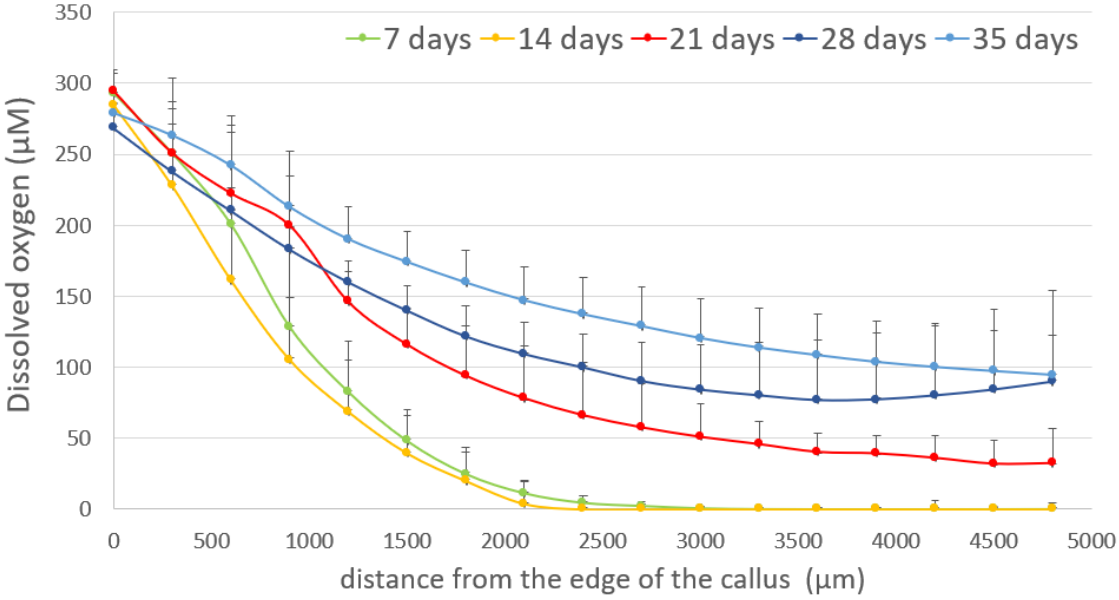
Fig. 4. Damages induced by heat stress in *Vitis vinifera* Gamay Fréaux cell cultures and *Arabidopsis thaliana* Col-0 seedlings, as a function of pretreatments with various ethanol concentrations. Pigments released from Gamay cell cultures, absorbing at **A)** 420 nm, yellow range, **B)** 520 nm, red range and **C)** 620 nm, blue range, following a 47°C heat-stress (see material and methods for details). Results are means ± SE, n = 8 biological replicates, small letters indicate a significant difference ($p < 0.05$) between groups, multiple comparisons by Fisher's LSD. **D)** Injury index of Arabidopsis seedlings based on ion leakage measurements following a 43°C heat-stress (see material and methods for details). Results means + SE, n = 7 biological replicates, small letters indicate a significant difference ($p < 0.05$) between groups, multiple comparisons by Tukey's HSD.



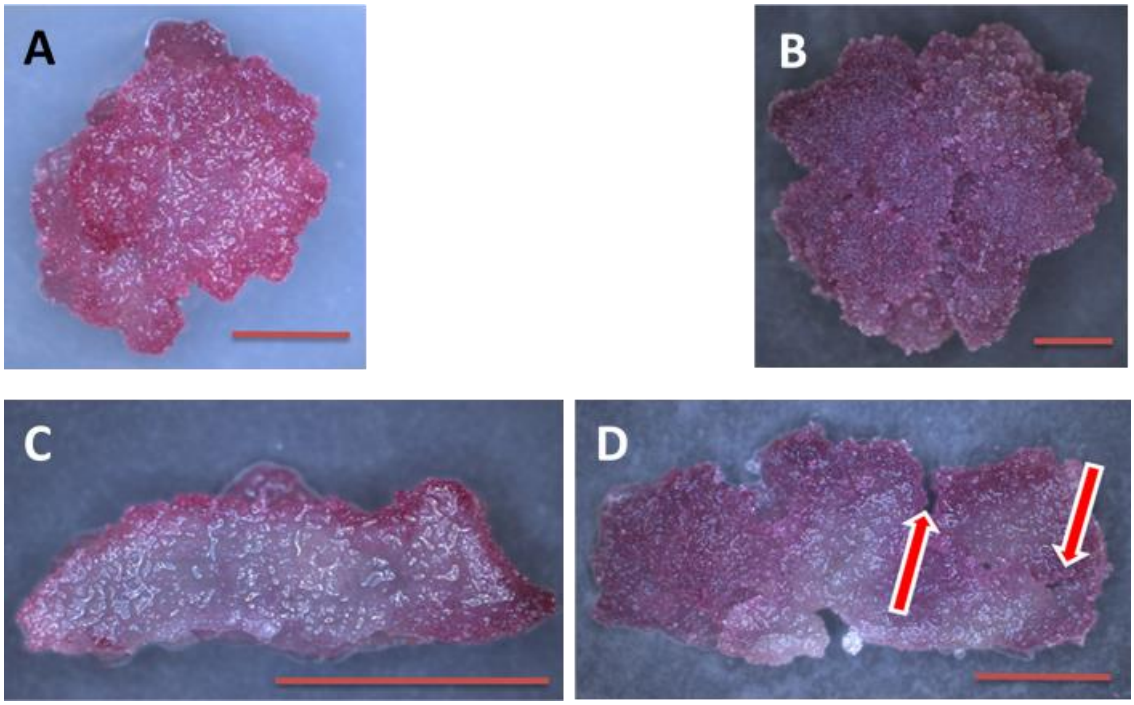
Supplementary Fig. S1. Heat-stress monitoring in Gamay cell cultures. The heat stress was applied in an incubator under dark conditions. The data represent the average temperature ± SD at the surface of the growth medium (n=3).



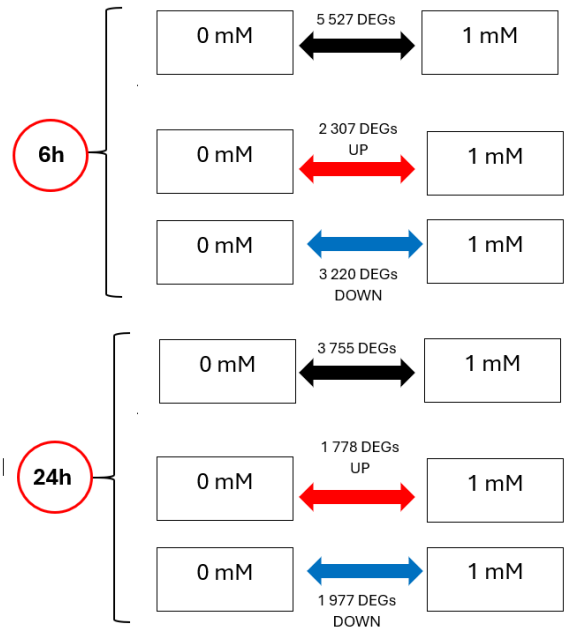
Supplementary Fig. S2. Average weight of a callus of *Vitis vinifera* cv. Gamay as a function of development time after sub-culturing. n = 12 different calli, error bars show SD, different small letters indicate significant differences at 0.05 (Dunn's test).



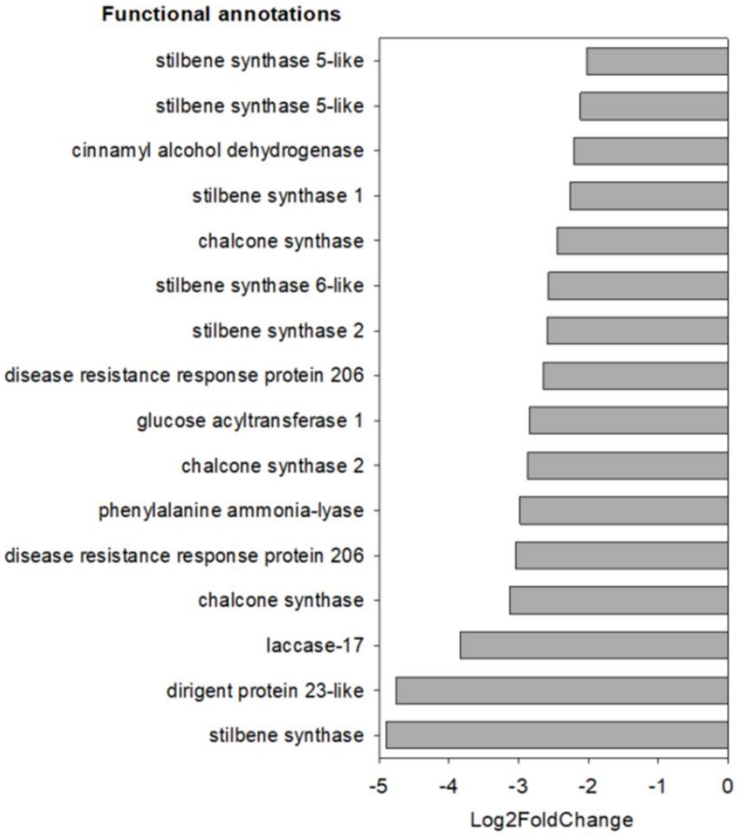
Supplementary Fig. S3. Dissolved O_2 profiles of *Vitis vinifera* L. cv. Gamay Fréaux cell cultures as a function distance from the edge of the callus (in µm) and the development time (7, 14, 21, 28 and 35 days), n = 4 different calli, error bars show SD.



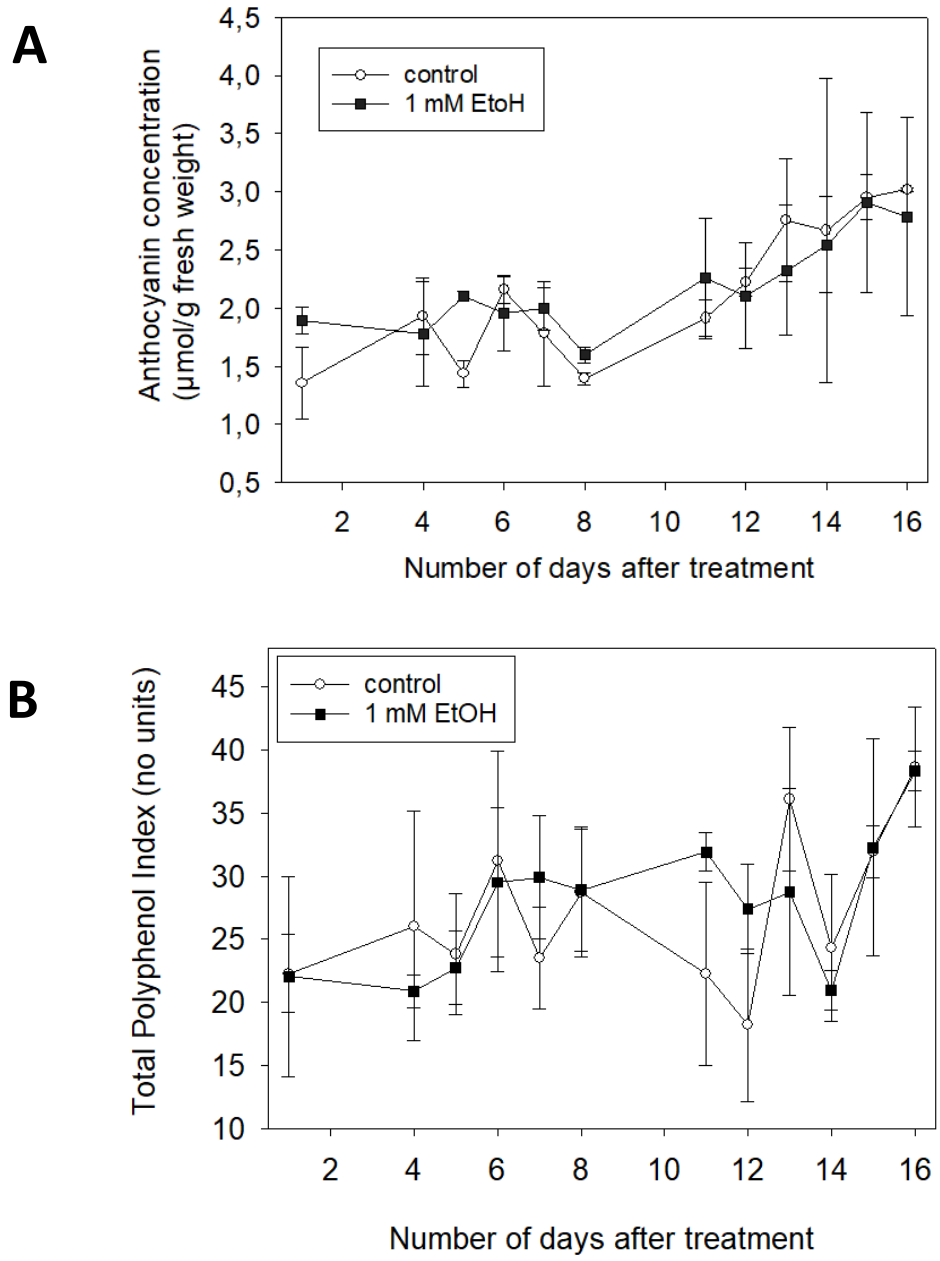
Supplementary Fig. S4. Photographs of calli seen from above (**A** and **B**) and photographs of calli cross-sections (**C** and **D**). Calli are either 14 days (**A** and **C**) or 35 days (**B** and **D**) old for comparison. Scale: the orange bar represents 0.5 cm. Red arrows show invaginations and gaseous interstices.



Supplementary Fig. S5. Transcriptional dynamics showing the number of DEGs upregulated and downregulated, 6 and 24 hours after a 1 mM ethanol treatment, compared to the controls. DEGs shown in this figure are all the genes with a p-adj < 0.05, with no regard to their Log2FoldChange.



Supplementary Fig. S6. Details of the 16 DEGs, within the enriched GO term "secondary metabolism process", ordered by their respective fold changes, comparing 1 mM ethanol treatment to control, at 6 hour post-treatment.



Supplementary Fig. S7: Temporal changes in **A)** anthocyanin concentration and **B)** in the total polyphenol index (TPI) in grapevine cell cultures following an initial treatment of 1 mM ethanol (black squares) or a control treatment (white circles). Data represent the mean ± SD of three samples.

Supplementary Table S1. Composition of growth medium

for *Vitis vinifera* Gamay Fréaux cell cultures *(as it is not easy to find in old articles)*

Macronutrients	mg/L
KNO_3	2,500
$(NH_4)_2SO_4$	150
$CaCl_2$, $2H_2O$	150
NaH_2PO_4, $2H_2O$	150
$MgSO_4$, $7H_20$	250
Microelements	mg/L
$MnSO_4$, H_2O	16.9
$ZnSO_4$, $7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$NaMoO_4$, $2H_2O$	0.25
Cu SO_4, $5H_2O$	0.025
$CoCl_2$, $6H_2O$	0.025
Iron/EDTA	mg/L
FeSO4, 7H2O	27.85
Na2EDTA	37.85
Vitamins	mg/L
Myoinositol	100
D-pantothenic acid, hemicalcium salt	1
Biotin	0.01
Nicotinic acid	1
Thiamine-HCl	1
Pyridoxine-HCl	1
Organic compounds	mg/L
Sucrose	20,000
Casein hydrolate	250
Growth regulators	mg/L
Kinetin	0.2
1-naphthaleneacetic acid	0.1
Agar	9 g/L
NaOH	Adjust to pH = 6

Supplementary Table S2. Table showing the 'concentration-effect' of the ethanol treatment in 3-week-old calli, at the time of treatment with the exogenous ethanol solution. The endogenous ethanol concentration was measured in 3 calli. The final ethanol concentration after treatment was calculated to account for the light ethanol change generated by the 1 mM EtOH treatment.

Addition of 100µl of [EtOH] µM	[EtOH] in callus measured before treatment (µM)	Calculated [EtOH] in callus after treatment (µM)	EtOH gain (µM) at the callus level	Calculated variation in [EtOH](%)
1000	406 ± 2	460	+ 54	+ 13,3

N.B: The addition of 100 µl of an ethanol solution at 1mM results in a calculated final concentration in the callus of 460 µM, i.e. an increase of +13.3% compared to an untreated callus.

Supplementary Table S3A. Quality of the alignment - Percentage of uniquely mapped reads on the *Vitis vinifera* cv. Chasselas reference genome (Djari *et al*., 2024).

Treatment duration	sample designation	treatment	% of uniquely mapped reads
6h	etha_6h_0mM_1	control	93.12
6h	etha_6h_0mM_2	control	93.14
6h	etha_6h_0mM_3	control	92.48
6h	etha_6h_1mM_2	Ethanol 1 mM	93.51
6h	etha_6h_1mM_3	Ethanol 1 mM	92.89
6h	etha_6h_1mM_4	Ethanol 1 mM	93.45
24h	etha_24h_0µM_2	control	92.55
24h	etha_24h_0µM_3	control	92.48
24h	etha_24h_0µM_4	control	92.09
24h	etha_24h_1000µM_1	Ethanol 1 mM	92.44
24h	etha_24h_1000µM_2	Ethanol 1 mM	92.96
24h	etha_24h_1000µM_3	Ethanol 1 mM	92.84

Supplementary Table S3B. Assignment rate to features on the *Vitis vinifera* cv. Chasselas reference genome (Djari *et al*., 2024).

Treatment duration	sample designation	treatment	% of reads assigned to features
6h	etha_6h_0mM_1	control	83.40
6h	etha_6h_0mM_2	control	83.07
6h	etha_6h_0mM_3	control	81.77
6h	etha_6h_1mM_2	Ethanol 1 mM	83.01
6h	etha_6h_1mM_3	Ethanol 1 mM	82.11
6h	etha_6h_1mM_4	Ethanol 1 mM	82.95
24h	etha_24h_0µM_2	control	80.69
24h	etha_24h_0µM_3	control	82.45
24h	etha_24h_0µM_4	control	80.93
24h	etha_24h_1000µM_1	Ethanol 1 mM	82.08
24h	etha_24h_1000µM_2	Ethanol 1 mM	83.56
24h	etha_24h_1000µM_3	Ethanol 1 mM	81.12

Supplementary Tables S4 to S9 (Excel files) available on the publisher website